CpG Island Methylation of DNA Damage Response Genes in Advanced Ovarian Cancer

Jens M. Teodoridis,[1] Jacqueline Hall,[1] Sharon Marsh,[4] Hilary D. Kannall,[4] Catriona Smyth,[1] Jorge Curto,[1] Nadeem Siddiqui,[2] Hani Gabra,[3] Howard L. McLeod,[4] Gordon Strathdee,[1] and Robert Brown[1]

[1]Centre for Oncology and Applied Pharmacology, Cancer Research UK Beatson Laboratories, University of Glasgow; [2]Department of Gynaecological Oncology, University of Glasgow, Glasgow Royal Infirmary, Glasgow, United Kingdom; [3]Department of Medical Oncology, University of Edinburgh, Western Infirmary, Edinburgh, United Kingdom; and [4]Department of Medicine, Washington University in St. Louis, St. Louis, Missouri

Abstract

We have determined the methylation frequencies of 24 CpG islands of genes associated with DNA damage responses or with ovarian cancer in 106 stage III/IV epithelial ovarian tumors. We have analyzed this data for whether there is evidence of a CpG island methylator phenotype or associations of CpG island methylation with response to chemotherapy in advanced ovarian cancer. Frequent methylation was observed for *OPCML, DCR1, RASSF1A, HIC1, BRCA1,* and *MINT25* (33.3%, 30.7%, 26.4%, 17.3%, 12.3%, and 12.0%, respectively), whereas no methylation was observed for *APAF-1, DAPK, FANCF, FAS, P14, P21, P73, SOCS-3,* and *SURVIVIN*. The remaining genes showed only a low frequency of methylation, <10%. Unsupervised gene shaving identified a nonrandom pattern of methylation for *OPCML, DCR1, RASSF1A, MINT25, HIC1,* and *SFRP1,* supporting the concept of concordant methylation of these genes in ovarian cancer. Methylation of at least one of the group of genes involved in DNA repair/drug detoxification (*BRCA1, GSTP1,* and *MGMT*) was associated with improved response to chemotherapy (P = 0.013). We have examined the frequency of a polymorphism in the DNA methyltransferase gene *DNMT3b6,* which has been previously reported to affect gene transcription and cancer risk. The genetic polymorphism in the *DNMT3b6* gene promoter (at position −149) is not significantly associated with the concordant methylation observed, but is weakly associated with the overall frequency of methylation at the genes examined (P = 0.04, n = 56). This supports the hypothesis that genetic factors affecting function of DNMT genes may underlie the propensity of tumors to acquire aberrant CpG island methylation. (Cancer Res 2005; 65(19): 8961-7)

Introduction

Hypermethylation of CpG islands located within or close to the 5′ region of genes is associated with transcriptional repression of these genes (1). Aberrant methylation of multiple CpG islands is a frequent event in epithelial ovarian cancer compared with normal ovarian surface epithelium (2). CpG island hypermethylation of genes such as *BRCA1, RASSF1A,* and *OPCML,* among others, is a relatively early event in ovarian tumorigenesis (3, 4). Clusters of CpG islands become comethylated in cancers, including late-stage ovarian cancer, suggesting the existence of groups of genes defining particular CpG island methylator phenotypes of ovarian cancer that are independent of histologic type (5, 6). However, the concept of tumors having a CpG island methylator phenotype has recently been questioned (7, 8). To further elucidate the role of CpG island methylation in ovarian cancer, we have determined the methylation frequencies of 24 CpG islands (*APAF-1, BLU, BRCA1, CASP8, DAPK, DCR1, FANCF, FAS, GSTP1, HIC1, MGMT, MINT25, MLH1, OPCML, P14, P16, P21, P73, PTEN, RASSF1A, SFRP1, SOCS-3, SURVIVIN,* and *TMS1*) in 106 stage III/IV epithelial ovarian tumors.

To address whether a CpG island methylator phenotype may exist in a subset of ovarian tumors, unsupervised gene shaving (9) was used to identify coherent patterns of methylation that separate the samples into subgroups. The primary aim of this approach was to investigate the coherence of gene methylation and identify patterns of methylation that are nonrandom in this group of late-stage ovarian tumors. As a secondary question, we investigated if the pattern represented an underlying process which was independent of age, histologic subtype, or clinical outcome.

Many of the genes we have examined are involved in DNA damage responses such as cell cycle control, apoptosis, and DNA repair (see Table 1). Because such DNA response pathways have been shown in experimental models to be associated with resistance or sensitivity to DNA damaging agents, absence of specific proteins due to methylation-associated transcriptional silencing may affect response of tumors to chemotherapy (10). Disruption of any constituent of a given DNA damage response pathway has the potential to affect the functioning of that cellular response; therefore, we examined potential associations of methylation with response to chemotherapy using gene groupings based on known gene function. As a separate analysis, we applied partially supervised gene shaving (9) to try and identify a pattern of methylation that correlated with response.

DNA methylation involves the enzymatic addition of a methyl group to the carbon-5 position of cytosine in DNA at CpG dinucleotides (11). DNMT1 is believed to function primarily to maintain the DNA methylation pattern following the synthesis of new DNA during cell division, as it exhibits much higher activity on hemimethylated DNA than on unmethylated DNA (12). DNMT3a and 3b show no preference for hemimethylated DNA and, based on inactivation of the *DNMT3a* and *3b* genes in mice, they are believed to function principally as *de novo* methyltransferases (13). Mutations in *DNMT3b* can give rise to the recessive autosomal disorder ICF syndrome, which has abnormalities in

Note: H. Gabra is currently at the Department of Cancer Medicine, Hammersmith Hospital, Imperial College London, London W12 0HS, United Kingdom.

Requests for reprints: Robert Brown, Department of Medical Oncology, Cancer Research UK Beatson Laboratories, Garscube Estate, Switchback Road, G61 1BD Glasgow, Scotland. Phone: 011-141-330-4335; Fax: 011-141-330-4127; E-mail: r.brown@beatson.gla.ac.uk.

doi:10.1158/0008-5472.CAN-05-1187

Table 1. Methylation frequencies in stage III/IV tumors

Gene	Function	MSP primer reference	Methylation frequency	
			Stage III	Stage IV
APAF-1	Apoptosis	(48)	0	0
BLU		(49)	1.2% (1 of 80)	0
BRCA1	DNA repair	(5)	8.8% (7 of 80)	23.1% (6 of 26)
CASP8	Apoptosis	(50)	1.2% (1 of 80)	0
DAPK	Apoptosis	(51)	0	0
DcR1	Apoptosis	(52)	28.1% (16 of 57)	38.9% (7 of 18)
FancF	DNA repair	(34)	0	0
Fas	Apoptosis	(53)	0	0
GSTp1	Detoxification	(54)	1.2% (1 of 80)	0
HIC1		(55)	10.5% (6 of 57)	38.9% (7 of 18)
MGMT	DNA repair	(51)	0	3.8% (1 of 26)
MINT25		(5)	12.3% (7 of 57)	11.1% (2 of 18)
MLH1	DNA repair/apoptosis	(19)	5% (4 of 80)	7.7% (2 of 26)
OPCML	Ovarian tumor suppressor	(4)	36.8% (21 of 57)	22.2% (4 of 18)
P14	Apoptosis	(27)	0	0
P16	Cell cycle	(25)	0	3.8% (1 of 26)
P21	Cell cycle		0	0
P73	Apoptosis	(5)	0	0
PTEN	Proliferation		8.6% (5 of 58)	0
RASSF1A	Apoptosis	(56)	26.2% (21 of 80)	26.9% (7 of 26)
SFRP-1	Proliferation		5.2% (3 of 58)	5.6% (1 of 18)
SOCS-3	Proliferation		0	0
Survivin	Apoptosis		0	0
TMS1	Apoptosis	(57)	5.1% (3 of 59)	5.6% (1 of 18)

methylation including hypomethylation of CpG islands (14). DNMT3b exists in multiple isoforms depending either on promoter usage or splice variants. A −149 C>T polymorphism in the promoter of the *DNMT3b6* isoform has been previously associated with increased risk of lung cancer and poor overall survival of head and neck squamous cell carcinoma (15–17). Trancriptional activity at the *DNMT3b6* gene has been suggested to be reduced due to the thymidine mutation at this site (15–17). We have examined whether the −149 C>T polymorphism in the promoter of the *DNMT3b6* gene (15, 16) correlates with methylation status in these late-stage ovarian tumors.

Materials and Methods

Patient samples. Biopsies from 106 stage III/IV ovarian surface epithelial tumors were collected after surgical removal at presentation before chemotherapy. Ethical approval for all samples collected was obtained and samples were collected according to Medical Research Council operational and ethical guidelines on "Human tissue and biological samples for use in research." All samples were stored frozen at −70°C. Pathology reports, including histologic subtype and grade, were obtained where possible. Response to therapy, defined by lesion size, was obtained from the patient's case notes in a retrospective manner. This was done in an anonymized fashion by data managers from the Beatson Oncology Centre and Stobhill Hospital (Glasgow), who were blind to methylation status. Response was defined by Modified Southwest Oncology Group criteria as previously described (18). Response to chemotherapy was measured in all patients that had evaluable disease (i.e., had detectable disease following cytoreductive surgery before chemotherapy). No significant differences in proportions of patients with gene methylation were observed between patients with evaluable and nonevaluable disease (data not shown). All patients were treated with either cisplatin or carboplatin and the majority were also treated with a taxoid (69%).

Methylation-specific PCR. Genomic DNA was extracted for methylation analysis as previously described (19). *In vitro* methylated DNA (Intergen, Oxford, United Kingdom), DNA from male whole blood (Promega, Southampton, United Kingdom), and DNA from normal and immortalized ovarian surface epithelium (kindly provided by Dr. T. Huang, Ohio State University, Columbus, OH) were used as controls. One microgram of genomic DNA was modified with sodium bisulfite using the CpGenome DNA Modification Kit (Intergen) according to the specifications of the manufacturer. After modification, the DNA was eluted into 40 μL Tris-EDTA. Methylation-specific PCR (MSP) was done in a total volume of 25 μL, containing 1 μL modified template DNA, 150 ng of each primer (TAGN, Gateshead, United Kingdom), 0.2 mmol/L deoxynucleotide triphosphates (Applied Biosystems, Warrington, United Kingdom), and 1 unit FastStart Taq (Roche Diagnostics, Lewes, United Kingdom). MSP reactions were subjected to initial incubation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, and annealing at the appropriate temperature for 30 seconds and 72°C for 30 seconds. Final extension was done by incubation at 72°C for 5 minutes. Primer sequences and MSP conditions are detailed in methPrimerDB (http://medgen.ugent.be/methprimerdb/index.php). MSP products were separated on 2% agarose gels and visualized after ethidium bromide staining.

Although MSP is a sensitive and widely used assay for the analysis of methylation patterns in tumors, care has to be taken to minimize false-positive and false-negative PCR products (20). No PCR product using methylation-specific primers, and hence no evidence of methylation of these CpG islands, was observed for normal or immortalized ovarian surface epithelial cells or DNA extracted from peripheral blood. In all cases, MSP gave a product using *in vitro* methylated DNA. To avoid confounding effects of low levels of unmodified DNA, the number of cycles of PCR used did not exceed 35 cycles; for subsequent analysis, we have disregarded very low intensity signals. All MSP data have been done on at least two independent modifications of DNA. We have not scored any signal that was weaker than

the signal obtained with a 1:10 dilution of *in vitro* methylated DNA into normal unmethylated peripheral blood DNA or any signal that was not detectable in repeated experiments. False-positive MSP results can be obtained if the bisulfite modification is not complete (20). To verify successful bisulfite modification of the DNA, a sequence that contains cytosines was amplified after bisulfite modification with primers that will only give an amplified product if the cytosines in the template sequence have been successfully converted to uracils. For this purpose, a region of the *CALPONIN* promoter region was amplified with every modified DNA sample. Samples that did not give a band of similar intensity were considered unmodified or incompletely modified and the modification reaction was repeated for those samples. In addition, the methylation status of CpG islands that frequently gave positive MSP results was confirmed by COBRA, and there was >90% concordance between the MSP and COBRA results.

Combined bisulfite restriction analysis. Combined bisulfite restriction analysis (COBRA; ref. 21) of *BRCA1, HIC1, MLH1, OPCML, RASSF1A, SFRP1,* and *TMS1* was done by amplifying bisulfite-modified promoter regions with primers that do not discriminate between methylation states. Methylation levels were assessed by subsequent restriction digest of the amplified products. Primers, amplification conditions, and restriction enzymes used are detailed in methPrimerDB (http://medgen.ugent.be/methprimerdb/index.php).

Statistical and bioinformatic analysis. For 70 of the 106 late-stage ovarian cancer samples, clinical data on response to chemotherapy were available. Genes were grouped according to their biological role in certain pathways involved in cancer because we hypothesized that disruption of any constituent of the pathway has the potential to affect the functioning of that cellular response. Grouping genes into predefined groups also reduces problems of multiple analyses. A group of genes was considered methylated if at least one gene within that group was methylated. Response was defined as complete or partial clinical response. Correlation between methylation and response was assessed by the χ^2 test unless the smallest expected value is <5—then Fisher exact test was used. Correlation between methylation and *DNMT3b6* polymorphism status was analyzed by the Mann-Whitney test. Statistical analyses were done with SPSS 11.0.

To investigate patterns of coherent methylation, unsupervised gene shaving was applied to 106 stage III/IV samples and 23 genes; modifications from the original algorithm were used for cluster selection. *SOCS-3* was removed from the analysis. Three tumor samples were excluded as <50% of the markers were analyzed; all other genes had no more than 30% data missing. Missing values were imputed using the K nearest neighbor algorithm (9, 22).

To investigate patterns of concordant methylation that correlate with response to chemotherapy, partially supervised gene shaving was applied to 70 stage III/IV samples, for which response information was available, and 23 genes. The level of supervision was selected as a compromise between good correlation of the pattern score with the difference in means in the two response categories and maintaining a high percentage of variance explained by the cluster ($\alpha = 0.6$). The same modified procedures as for unsupervised gene shaving were applied.

Modifications to gene shaving. We used a cluster quality score (R^2) from the gap statistical calculation (9) to identify clusters of better quality than could have been achieved by chance. A large value of R^2 reflects a pattern of coherence between genes but which differs across samples. If the R^2 score is outside the upper confidence interval of the 97.5% quantile of the randomized data R^2 score distribution (200 permutations), the pattern was unlikely to have occurred by chance. The confidence limits were estimated by bootstrapping (300 replications). We also calculated the variance explained by the gene shaving pattern score with respect to full Principal Components Analysis. To test associations of the pattern with response to chemotherapy, histology, and *DNMT3b6* polymorphism, the pattern score (in this case representing the frequency of gene methylation) was used in logistic regression; correlation between methylation patterns and age was assessed by linear regression.

As a subsequent question, we asked if methylation of any gene in the pattern was associated with response based on the premise that methylation of any one gene in the pattern means the patient has a methylator phenotype. To test this methylator phenotype association with chemotherapy, histology, age, and *DNMT3b6* polymorphism, the χ^2 test was used. Gene shaving was done in R v2.0.0 (23). Original gene shaving code was developed by Kim-Anh Do and Sijin Wen (http://lib.stat.cmu.edu).

***DNMT3b6* polymorphism analysis.** Genotyping for *DNMT3b6* −149 C>T was carried out using DNA from 58 stage III/IV ovarian tumors, for which appropriate ethical permission was granted, using PCR and pyrosequencing. PCR was done with primers designed using Primer Express version 2.0 (ABI, Foster City, CA), forward 5′-AGTTGTCCTGAAGCTGGCTACCA-3′ and reverse 5′-Biotin-GTTCGGACCTAGAAGCAAGAGAGG-3′, at 58°C annealing temperature. The pyrosequencing primer, 5′-CCCCGCCAGACCC-3′, was designed using pyrosequencing SNP Primer Design Version 1.01 software (http://techsupport.pyrosequencing.com). Pyrosequencing was carried out using the pyrosequencing PSQ hs96A instrument and software (Biotage, Uppsala, Sweden), as previously described (24). Hardy-Weinberg equilibrium was determined using HWsim (http://info.med.yale.edu/genetics/kkidd/programs.html).

Results

CpG island methylation in epithelial ovarian tumors. We have analyzed CpG island methylation in epithelial ovarian tumor DNA using MSP (25), and confirmed positive MSP results for genes that were frequently methylated by COBRA (21). Primers for MSP were designed to detect methylated sequences and chosen based on previous studies correlating methylation of these regions of the CpG island with transcriptional silencing (Table 1). We analyzed the methylation status of the CpG islands associated with the promoter/first exon of 24 genes (*APAF-1, BLU, BRCA1, CASP8, DAPK, FANCF, FAS, GSTP1, MGMT, MLH1, P14, P16, P21, P73, RASSF1A, SOCS-3, DCR1, HIC1, MINT25, OPCML, PTEN, SFRP1, TMS1,* and *SURVIVIN*) in 106 stage III/IV ovarian epithelial tumors that were taken at presentation before chemotherapy (see Table 1). These include genes that are involved in cellular responses to DNA damage, such as genes involved in DNA repair (*BRCA1, FANCF, GSTPI,* and *MGMT*), regulators of proliferation (*P16, P21, PTEN, SFRP1,* and *SOCS-3*), regulators of apoptosis (*APAF-1, CASP8, DAPK, DCR1, FAS, MLH1, P14, P73, RASSF1A, SURVIVIN,* and *TMS1*), and genes that can become methylated in ovarian cancer (*HIC1, MINT25,* and *OPCML*). The results are summarized in Table 1 and examples of MSP shown in Fig. 1. As described in Materials and Methods, we have used stringent criteria for scoring a sample positive and have disregarded low levels of methylation (MSP signals <1:10 dilution of *in vitro*

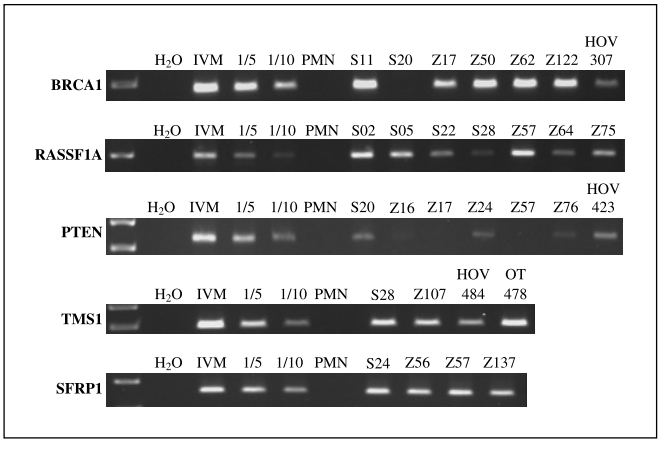

Figure 1. Examples for typical MSP results. *H_2O,* reaction without template DNA; *IVM,in vitro* methylated DNA; *1/5* and *1/10*, dilutions of IVM in PMN; *PMN,* DNA from male whole blood.

methylated DNA into normal unmethylated DNA). Methylation of at least one gene was observed in 60.4% stage III/IV ovarian tumor samples. Frequent methylation was observed for *OPCML, DCR1, RASSF1A, HIC1, BRCA1,* and *MINT25* (33.3%, 30.7%, 26.4%, 17.3%, 12.3%, and 12.0%, respectively), whereas no methylation was observed for *APAF-1, DAPK, FANCF, FAS, P14, P21, P73, SOCS-3,* and *SURVIVIN*. The remaining genes showed only a low frequency of methylation, <10%.

To examine concordant methylation in the current study, we have used a bioinformatics approach, gene shaving (26). Pattern 1 (Fig. 2*A*) was identified, which has a cluster score (R^2) of 0.34 and accounts for 87.1% of the Principal Component Analysis (PCA) solution. The derivation of pattern 1 is unlikely to have occurred by chance as shown by the distribution of the cluster quality R^2 statistic for 200 permutations (Fig. 2*B*). Pattern identification ceased here as the next pattern only accounted for a small proportion of the full PCA solution (<3%). Pattern 1 shows concordant positive methylation between genes and is mostly comprised of frequently methylated genes *OPCML1, DCR1, RASSF1A, MINT25,* and *HIC1,* and also *SFRP1,* which shows less methylation. No CpG islands were identified in the pattern to have negative concordance, which the gene shaving approach would have identified if present. One frequently methylated gene which is notable in its absence from this pattern is *BRCA1,* suggesting methylation of this gene may occur via a different underlying process or biological selection, consistent with previous observation using a different analysis on a different group of stage III/IV ovarian tumors (5). The methylation pattern from unsupervised gene shaving was independent of age ($P = 0.239$, $n = 100$) and histologic subtype ($P = 0.247$, $n = 80$). The clinical characteristics of the patients that are methylated at any one of the genes showing concordant methylation compared with those showing no methylation at any of these genes are shown in Table 2.

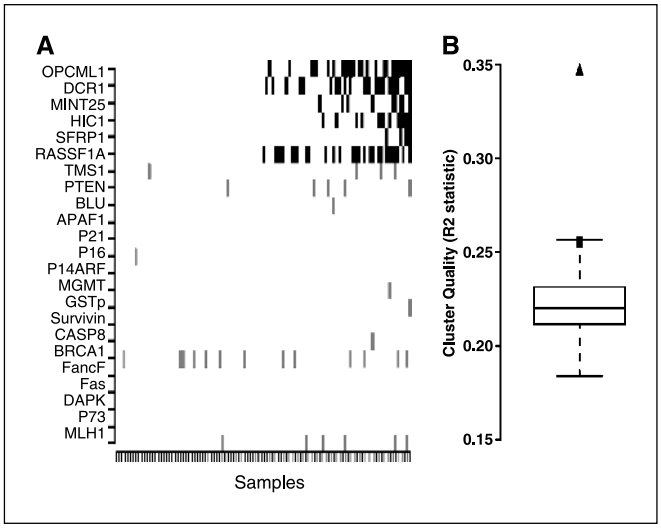

Figure 2. Gene shaving analysis to identify comethylated CpG islands. *A,* the pattern of gene methylation (*black*) identified by unsupervised gene shaving separates patients using a comethylated subsample of all methylation events (*shading*). Samples are ordered from right to left according to the frequency (number of methylation events per sample) of methylation in the pattern. This cluster explains 87.1% of the equivalent principal components analysis solution ($n = 106$). The first principal component accounts for 28.7% of the data variance. *B,* the box plot shows the distribution of the cluster quality R^2 statistic for 200 permutations. □, upper confidence interval of the distribution; △, R^2 value of pattern 1. The total variance (VT = 0.12) of a cluster is the summation of two measures between cluster variance (VB = 0.04) representing subsample separation and within-cluster variance (VW = 0.8) representing coherence between genes in the sample. R^2 is the proportion of variance explained by the cluster (VB / VT = 0.344); a larger R^2 suggests a tighter cluster of coherent genes.

Table 2. Clinical characteristics of patients

	Unmethylated at any one of pattern*	Methylated at any one of pattern*
	n	*n*
Stage		
III	42	38
IV	11	15
Response		
Complete/partial response	22	26
Stable/progressive disease	12	12
Not evaluable	21	15
Chemotherapy		
Platinum	17	21
Platinum/taxoid	31	27
Histopathology		
Serous	28	27
Nonserous	15	10
Age	Median, 58 Range, 19-82	Median, 62 Range, 31-81

*Methylated at any one or unmethylated at all of the genes in methylation pattern identified from gene shaving (*OPCML1, DCR1, RASSF1A, MINT25, HIC1,* and *SFRP1*).

Correlation with response to chemotherapy. Because the majority of the genes examined have been associated in experimental models with cellular sensitivity to DNA damage (10), we examined if methylation patterns correlate with response to chemotherapy. All of the patients have been treated with platinum-based chemotherapy (cisplatin or carboplatin) and the majority with a taxoid (69%). Only a subgroup of patients were evaluable for response and, for the purpose of analysis, complete and partial clinical response have been combined and compared with stable and progressive disease. The CpG islands were grouped according to the function of the corresponding gene or according to the unsupervised clusters identified above and examined for associations with clinical response to chemotherapy. For the grouping based on function, a group was considered methylated if at least one of its members was methylated. Methylation of any one of the 24 CpG islands examined compared with no gene methylated did not associate with response in those patients that were evaluable ($P = 0.51$, $n = 64$). Methylation of the group of genes involved in regulation of apoptosis (*APAF-1, CASP8, DAPK, DCR1, FAS, MLH1, P14, P73, RASSF1A, SURVIVIN,* and *TMS1*) was not significantly associated with response to chemotherapy in those patients that were evaluable ($P = 0.74$). Likewise, methylation of the groups of genes involved in cell cycle control and proliferation (*P16, P21, PTEN, SFRP1,* and *SOCS-3*) did not significantly correlate with response ($P = 0.999$). However, methylation of at least one gene involved in DNA repair (*BRCA1, GSTP1,* and *MGMT*) was significantly associated with increased response to chemotherapy ($P = 0.013$, $n = 70$). In the patient group that showed methylation of *BRCA1, GSTP1* or *MGMT,* response rate to chemotherapy was 100%

as compared with the response rate of 62.7% for patients not showing methylation of one of these genes. Because the majority of the tumors in the DNA repair group have *BRCA1* methylated (85%) subsequent to the analysis described, we examined whether methylation of *BRCA1* was associated with response, and it was of borderline significance ($P = 0.049$, $n = 70$).

The pattern identified by unsupervised gene shaving, pattern 1 (representing the frequency of methylation of the group of genes identified), was independent of response to chemotherapy ($P = 0.294$, $n = 70$). Partially supervised gene shaving was used to find patterns that associate with response to chemotherapy. A single pattern was identified that included 10 genes, *BRCA1, GSTP1, RASSF1A, PTEN, SFRP1, TMS1, HIC1, MINT25, DCR1,* and *OPCML*. Pattern identification ceased after this as the next cluster identified <5% of the equivalent full PCA solution. Based on a permutation test, the pattern identified was unlikely to have occurred by chance ($P = 0.005$); however, the pattern did not significantly associate with response to chemotherapy ($P = 0.078$, $n = 70$) using logistic regression.

Methylation status is associated with *DNMT3b6* genotype. The −149 C>T polymorphism in the promoter region of the *DNMT3b6* isoform of the *de novo* DNA methyltransferase DNMT3b has previously been suggested to affect transcription levels of this gene (16). Furthermore, this polymorphism has previously been associated with increased risk of lung cancer and poor overall survival of head and neck squamous cell carcinoma (15–17). We have determined the genotype of the −149 polymorphism in 56 ovarian tumors that have been characterized for CpG island methylation. Due to limitations on ethical approval and amounts of DNA available, we were only able to do this on a subset of the tumors examined for methylation. So far, this is the only gene polymorphism in the DNMT family of genes we have examined. The frequency of the three genotypes in 58 samples is 16:30:12 (CC:CT:TT), which is in Hardy-Weinberg equilibrium, suggesting that this polymorphism does not alter the risk of developing ovarian cancer. As shown in Fig. 3, there is a significantly higher frequency of methylation at the CpG island examined in tumors with a CC genotype than in tumors with a T allele (TC or TT genotype; Mann-Whitney, $P = 0.04$, $n = 56$).

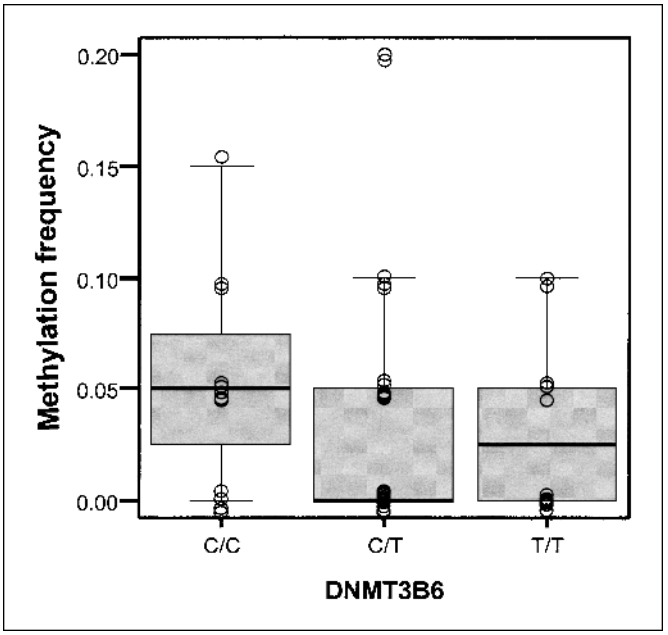

Figure 3. Box plot of methylation frequency for each *DNMT3b6* genotype. Methylation frequency is the number of methylation events per sample of the CpG islands examined.

Discussion

In this study, we analyzed the methylation frequencies of CpG islands at 24 genes in late-stage ovarian tumors. The analysis focused on genes that are involved in cellular responses to DNA damage, including genes involved in DNA repair, cell cycle control, and apoptosis signaling, as well as genes that can frequently become methylated in ovarian tumors. Previous studies had reported similar methylation frequencies in ovarian tumors for *BRCA1* (5, 27), *CASP8* (5), *DAPK* (28), *DCR1* (29), *HIC1* (5), *MINT25* (5), *MLH1* (5), *P16* (30), *P73* (31), *SFRP1* (32), and *SOCS-3* (33). Differing methylation frequencies had been reported for *FANCF, OPCML,* and *TMS1*, for which we observed methylation in 0%, 33.3%, and 5.2% of the late-stage ovarian tumors, respectively. Methylation of *FANCF* had been reported in 21% of ovarian tumors (34), but a considerably higher number of PCR cycles had been used in that study, which may explain the discrepancy, and some of the tumors in previous studies may represent germ cell tumors rather than epithelial ovarian tumors (35–38). A previous study reported methylation of *TMS1* in 19% of ovarian tumors but methylation frequencies varied between different tumor histologies and stages (28). This can, in part, explain the observed overall difference in methylation frequency because the proportions of the various histologies and stages in this study are different from the previous study. In agreement with the previous study, we observed *TMS1* methylation more frequently in clear cell ovarian tumors (100%). Methylation of *OPCML* had been observed in 83% of ovarian tumors (4), but tumor stage had not been indicated in that study. We observed higher *OPCML* methylation frequencies in borderline as well as early-stage tumors than in late-stage tumors;[5] therefore, different fractions of stages could possibly explain the observed differences in methylation frequencies. Another possible explanation could be that the *OPCML* methylation frequencies vary between different histologies and that the observed overall differences in methylation are due to different proportions of histologies.

For comparison with response, we grouped genes according to their functions or methylation clusters. The grouping of genes according to function is inevitably an oversimplification, but was decided before the analysis. Methylation of at least one gene involved in DNA repair or detoxification (*BRCA1, GSTP1,* or *MGMT*) was associated with higher response to chemotherapy ($P = 0.013$). MLH1 was not included in the "repair" group because although involved in repair of replication errors, it is not involved in repair of DNA damage induced by chemotherapeutic drugs. Indeed, lack of MLH1, rather than giving hypersensitivity due to lack of DNA damage repair, leads to DNA damage tolerance due to loss of engagement of an apoptotic response (39). MLH1 was therefore included in the "apoptosis" genes. The DNA repair enzyme MGMT removes mutagenic alkyl groups from the O^6 position of guanine, which could otherwise lead to G→A transitions after DNA replication (40). Methylation of a CpG island in the *MGMT* promoter is an independent predictor of longer survival for glioblastoma patients

[5] C. Smyth, J.M. Teodoridis, and R. Brown, unpublished data.

treated with a methylating agent (temozolomide), in addition to radiation, in a prospective study (41). Hypermethylation of the *MGMT* promoter also correlated with increased survival of patients with diffuse large B-cell lymphoma after chemotherapy which included cyclophosphamide (42). Glutathione-*S*-transferase (GST) proteins catalyze the conjugation of glutathione to a variety of electrophilic substrates, thereby enhancing the cell's ability to metabolize these potentially toxic substances and prevent the accumulation of compounds that can damage the DNA (43). Thus, loss of *GSTP1* expression cold lead to increased sensitivity to drugs, although there have been no studies thus far examining methylation of *GSTP1* and clinical chemosensitivity. Several *in vitro* studies show the relevance of the integrity of the BRCA1 pathway for tumor response to chemotherapy. It has been shown that BRCA1 deficiency is linked to sensitivity to cisplatin and other DNA damaging agents (44, 45), although the need for further clinical studies of BRCA1 and response to chemotherapy has recently been highlighted (46).

The current study is a retrospective study of a group of patients that have not all had the same chemotherapy. It will be important to confirm the observations and hypothesis generated in the present study in a prospective study of a more defined patient population. Such a study is currently under way through the Scottish Gynaecological Clinical Trials Group.

The mechanism underlying aberrant methylation of genes in cancer remains uncertain. Altered expression of the DNMT enzymes is a possible mechanism for the dysregulation of DNA methylation seen in tumors. DNMT3a and 3b could be thought to represent better candidates for causing the increased CpG island methylation observed in cancer cells due to their *de novo* methylation capability (15). However, conflicting studies indicating increased expression (23) or the absence of increased expression (22) of DNMT3a and 3b in tumor samples have been reported. Because genetic inactivation of DNMT1 and DNMT3b can lead to reversal of methylation in tumor cells (47), together with mutations in *DNMT3b* affecting expression and function of the protein (14, 16), we have examined association between a promoter polymorphism in *DNMT3b6* and frequency of gene methylation in the 24 genes examined. This particular polymorphism was chosen because previous studies had shown associations with increased risk of lung cancer and poor overall survival of head and neck squamous cell carcinoma (15–17). Previous studies have shown an increased risk for both the CT and TT genotypes compared with the CC genotype at this polymorphic site (16). The T allele has been previously associated with lower *DNMT3b6* transcription of this putative *de novo* DNMT (16). Based on these previous observations, we have combined the CT and TT genotypes in the analysis for comparison with the CC genotype. Consistent with a role for altered *DNMT3b6* expression in aberrant methylation, we observe reduced frequency of methylation in the 24 genes examined in stage III/IV ovarian tumors in the presence of at least one T allele. Although we see an apparent association with overall methylation frequency, we do not observe any association with the concordant methylation. Thus, tumors with methylation of any one of the genes in the concordant methylation pattern (Fig. 2) are not related to *DNMT3b6* polymorphism (P = 0.921). This is a relatively small hypothesis-generating study and the potential association between DNMT polymorphisms and methylation or clinical outcome is currently being examined in a much larger sample collection from the SCOTROC1 clinical trial (18).

Concordant methylation of genes has been observed in colon and gastric tumors, leading to the concept of some tumors having a CpG island methylator phenotype (6), and it has been postulated that genetic aberrations could pose one possible explanation for this phenotype (7). In epithelial ovarian cancer, previous studies have suggested two comethylated groups of genes (5): one group characterized by concordant methylation of *HIC1, MINT25, MINT31,* and *p73,* and the second group by methylation of *BRCA1,* which was negatively concordantly methylated with the first group. Recently, the concept of a CpG island methylator phenotype as characterized by concordant methylation of genes has been questioned (8). To examine concordant methylation in the current study, we have used a bioinformatics approach, gene shaving (26), which provides an objective approach to identify coherent patterns of methylation. We have identified concordant positive methylation between six CpG islands for *OPCML1, DCR1, RASSF1A, MINT25, HIC1,* and *SFRP1*, although CpG islands examined may have been falsely excluded from this grouping if methylation was infrequent due to the limitations of the gene shaving algorithm. Thus, we have identified a methylation pattern which may represent a nonrandom process whereby certain genes tend to be concordantly methylated, supporting the hypothesis that there is an underlying biological mechanism giving rise to comethylation of these genes. This pattern is independent of response to chemotherapy, histology, and age. Together these observations support the concept of a CpG island methylator phenotype in ovarian cancer.

Acknowledgments

Received 4/6/2005; revised 6/13/2005; accepted 7/6/2005.

Grant support: Cancer Research UK grants A2662 and A3286 and NIH grants GM63340 and R21 CA113491-01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Tim Huang and Dr. Susan Wei for kindly providing normal and immortalized ovarian surface epithelium DNA. We thank Andrea Hay, Jan Gorman, and the data managers at the Beatson Oncology Centre, Glasgow. We would also like to thank the patients for facilitating and consenting to the sample collection.

References

1. Bird A, Wolffe AP. Methylation-induced repression—belts, braces and chromatin. Cell 1999;99:451–4.
2. Wei SH, Chen CM, Strathdee G, et al. Methylation microarray analysis of late-stage ovarian carcinomas distinguishes progression-free survival in patients and identifies candidate epigenetic markers. Clin Cancer Res 2002;8:2246–52.
3. Ibanez de Caceres I, Battagli C, Esteller M, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. Cancer Res 2004;64:6476–81.
4. Sellar GC, Watt KP, Rabiasz GJ, et al. OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. Nat Genet 2003;34:337–43.
5. Strathdee G, Appleton K, Illand M, et al. Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes. Am J Pathol 2001;158:1121–7.
6. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–6.
7. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004;4:988–93.
8. Yamashita K, Dai T, Dai Y, et al. Genetics supersedes epigenetics in colon cancer phenotype. Cancer Cell 2003;4:121–31.
9. Hastie T, Tibshirani R, Botstein D, et al. Supervised harvesting of expression trees. Genome Biol 2001;2:RESEARCH0003.

10. Teodoridis JM, Strathdee G, Brown R. Epigenetic silencing mediated by CpG island methylation: potential as a therapeutic target and as a biomarker. Drug Resist Updat 2004;7:267–78.

11. Hendrich B, Bird A. Mammalian methyltransferases and methyl-CpG-binding domains: proteins involved in DNA methylation. Curr Top Microbiol Immunol 2000;249:55–74.

12. Bestor TH, Verdine GL. DNA methyltransferases. Curr Opin Cell Biol 1994;6:380–9.

13. Okano M, Bell DW, Haber DA, et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. Cell 1999;99:247–57.

14. Jiang YL, Rigolet M, Bourc'his D, et al. DNMT3B mutations and DNA methylation defect define two types of ICF syndrome. Hum Mutat 2005;25:56–63.

15. Lee SJ, Jeon HS, Jang JS, et al. DNMT3B polymorphisms and risk of primary lung cancer. Carcinogenesis 2005;26: 403–9.

16. Shen H, Wang L, Spitz MR, et al. A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer. Cancer Res 2002;62:4992–5.

17. Wang L, Rodriguez M, Kim ES, et al. A novel C/T polymorphism in the core promoter of human *de novo* cytosine DNA methyltransferase 3B6 is associated with prognosis in head and neck cancer. Int J Oncol 2004;25:993–9.

18. Vasey PA, Jayson GC, Gordon A, et al. Phase III randomized trial of docetaxel-carboplatin versus paclitaxel-carboplatin as first-line chemotherapy for ovarian carcinoma. J Natl Cancer Inst 2004;96:1682–91.

19. Strathdee G, MacKean M, Illand M, et al. A role for methylation of the *hMLH1* promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. Oncogene 1999;18:2335–41.

20. Rand K, Qu W, Ho T, et al. Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. Methods 2002;27:114–20.

21. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997;25:2532–4.

22. Hastie TR, Tibshirani R, Eisen MB, et al. Imputing missing data for gene expression arrays. Stanford University Statistics Department Technical report; 1999.

23. Ihaka R, Gentleman R. *R*: a language for data analysis and graphics. J Comput Graph Stat 1996;5:299–314.

24. Rose CM, Marsh S, Ameyaw MM, et al. Pharmacogenetic analysis of clinically relevant genetic polymorphisms. Methods Mol Med 2003;85:225–37.

25. Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.

26. Hastie T, Tibshirani R, Eisen MB, et al. "Gene shaving" as a method for identifying distinct sets of genes with similar expression patterns. Genome Biol 2000;1:RESEARCH0003.

27. Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. Eur J Cancer 2000;36:2294–300.

28. Terasawa K, Sagae S, Toyota M, et al. Epigenetic inactivation of TMS1/ASC in ovarian cancer. Clin Cancer Res 2004;10:2000–6.

29. Shivapurkar N, Toyooka S, Toyooka KO, et al. Aberrant methylation of trail decoy receptor genes is frequent in multiple tumor types. Int J Cancer 2004; 109:786–92.

30. Brown I, Milner BJ, Rooney PH, et al. NE inactivation of the p16INK4A gene by methylation is not a frequent event in sporadic ovarian carcinoma. Oncol Rep 2001;8: 1359–62.

31. Chen CL, Ip SM, Cheng D, et al. HY P73 gene expression in ovarian cancer tissues and cell lines. Clin Cancer Res 2000;6:3910–5.

32. Takada T, Yagi Y, Maekita T, et al. Methylation-associated silencing of the Wnt antagonist SFRP1 gene in human ovarian cancers. Cancer Sci 2004;95:741–4.

33. Sutherland KD, Lindeman GJ, Choong DY, et al. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. Oncogene 2004;23:7726–33.

34. Taniguchi T, Tischkowitz M, Ameziane N, et al. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med 2003;9: 568–74.

35. Aparicio A, Eads CA, Leong LA, et al. Phase I trial of continuous infusion 5-aza-2′-deoxycytidine. Cancer Chemother Pharmacol 2003;51:231–9.

36. Dhillon VS, Shahid M, Husain SA. CpG methylation of the FHIT, FANCF, cyclin-D2, BRCA2 and RUNX3 genes in Granulosa cell tumors (GCTs) of ovarian origin. Mol Cancer 2004;3:33.

37. Koul S, McKiernan JM, Narayan G, et al. Role of promoter hypermethylation in Cisplatin treatment response of male germ cell tumors. Mol Cancer 2004;3:16.

38. Narayan G, Arias-Pulido H, Nandula SV, et al. Promoter hypermethylation of FANCF: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. Cancer Res 2004;64:2994–7.

39. Luo Y, Lin FT, Lin WC. ATM-mediated stabilization of hMutL DNA mismatch repair proteins augments p53 activation during DNA damage. Mol Cell Biol 2004; 24:6430–44.

40. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 2004;4:296–307.

41. Hegi ME, Diserens AC, Godard S, et al. Clinical trial substantiates the predictive value of *O*-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Cancer Res 2004;10:1871–4.

42. Esteller M, Gaidano G, Goodman SN, et al. Hypermethylation of the DNA repair gene *O*(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. J Natl Cancer Inst 2002;94:26–32.

43. Tew KD, Ronai Z. GST function in drug and stress response. Drug Resist Updat 1999;2:143–7.

44. Sgagias MK, Wagner KU, Hamik B, et al. Brca1-deficient murine mammary epithelial cells have increased sensitivity to CDDP and MMS. Cell Cycle 2004;3:1451–6.

45. Tassone P, Tagliaferri P, Perricelli A, et al. BRCA1 expression modulates chemosensitivity of BRCA1-defective HCC1937 human breast cancer cells. Br J Cancer 2003;88:1285–91.

46. Kennedy RD, Quinn JE, Mullan PB, et al. The role of BRCA1 in the cellular response to chemotherapy. J Natl Cancer Inst 2004;96:1659–68.

47. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002;416:552–6.

48. Fu WN, Bertoni F, Kelsey SM, et al. Role of DNA methylation in the suppression of Apaf-1 protein in human leukaemia. Oncogene 2003;22:451–5.

49. Agathanggelou A, Dallol A, Zochbauer-Muller S, et al. Epigenetic inactivation of the candidate 3p21.3 suppressor gene BLU in human cancers. Oncogene 2003; 22:1580–8.

50. Teitz T, Wei T, Valentine MB, et al. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 2000; 6:529–35.

51. Balana C, Ramirez JL, Taron M, et al. O^6-Methylguanine-DNA methyltransferase methylation in serum and tumor DNA predicts response to 1,3-bis(2-chloroethyl)-1-nitrosourea but not to temozolamide plus cisplatin in glioblastoma multiforme. Clin Cancer Res 2003;9:1461–8.

52. van Noesel MM, van Bezouw S, Salomons GS, et al. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. Cancer Res 2002;62: 2157–61.

53. Hopkins-Donaldson S, Ziegler A, Kurtz S, et al. Silencing of death receptor and caspase-8 expression in small cell lung carcinoma cell lines and tumors by DNA methylation. Cell Death Differ 2003;10:356–64.

54. Jeronimo C, Usadel H, Henrique R, et al. Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. J Natl Cancer Inst 2001;93:1747–52.

55. Dong SM, Kim HS, Rha SH, et al. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. Clin Cancer Res 2001;7:1982–6.

56. Honorio S, Agathanggelou A, Wernert N, et al. Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular tumours and distinct methylation profiles of seminoma and non-seminoma testicular germ cell tumours. Oncogene 2003; 22:461–6.

57. Conway KE, McConnell BB, Bowring CE, et al. TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res 2000; 60:6236–42.